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Detection of Type-Specific Antigenic Determinants of Murine Mammary Tumor Viruses with Cell-Mediated Immune Assays (41323)

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Abstract. Exposure of lymphoid cells from normal and mammary tumor-bearing Balb/cCrgl mice to MMTV antigens result in positive responses in blastogenic transformation and migration inhibition reactions. The levels of these responses appear to depend on the murine strain source of the viral preparations. Enhanced reactivities were observed in cultures exposed to purified MMTV from culture fluids of mammary tumor cells of spontaneous origin in the C3H strain when compared to purified MMTV derived from milk of mice from the RIII strain. To ensure the virus relatedness of the differences observed, MMTVs obtained from a common source, i.e., the Crandall feline embryo kidney cell line, were employed in blastogenesis assays. Parallel testing of feline cell-derived C3H, RIII, and GR mammary tumor viruses resulted in different levels of [^{3}H]thymidine incorporation by spleen cells from Balb/cCrgl mice over a range of viral concentrations. These results indicate that it is possible to detect various types of MMTV variants with cell-mediated immune assays. The diverse functional reactivities elicited by these viruses may be related to the differences observed in their bioactivities as determined by incidence and development of mammary tumors and the potential of causing metastases.

Previous studies have shown that various isolates of mouse mammary tumor virus (MMTV) appear to differ in their biological activities (1, 2). Although these isolates cannot be morphologically distinguished there have been reports of differences in the sequences of their RNA genomes and in their modes of transmission (3–5). Using an immunodiffusion technique, Blair (6) demonstrated strain specificities in the mouse mammary tumor virion antigens. Recently, Teramoto *et al.* (7) were able to detect with competitive radioimmunoassays, type-specific antigens on gp52, the major external glycoprotein of high and low oncogenic murine mammary tumor viruses. In addition radioimmunoassays for the 36,000-dalton glycoprotein of MMTVs demonstrate type, group, and interspecies determinants (8). Recently, Squartini and Bistocchi (9) have found that foster nursing of virgin female Balb/c mice in the highly oncogenic C3H and RIII mouse strains resulted in differences in the levels of spontaneous mammary tumor incidence and the frequency of lung metastases.

In previous reports (10, 11) we have shown that lymphocytes from Balb/c mice, a mouse strain with a low incidence of spontaneous mammary tumors, were able to respond to MMTV antigens derived from RIII milk in blastogenesis and migration inhibition assays. In the present investigation we present evidence that the lymphoid cells of these mice have different levels of reactivities to the MMTVs from various mouse strains when tested in cell-mediated immune assays. Since the appearance and evolution of mammary tumors is influenced by virus-directed and host-controlled interactions, the diversity of MMTVs and their recognition by the lymphoid cells could explain the variations in mouse mammary tumor development and the differences in metastatic potential.

Materials and Methods. *Animals and tumors.* Balb/cCrgl mice were initially obtained from the Cancer Research Genetics Laboratory of the University of California. These mice have been maintained by brother–sister mating in our laboratories for the past 7 years. The syngeneic transplantable mammary tumor designated as D1-DMBA-3 was kindly provided by Dr. P. Blair (University of California) and maintained in our laboratories by subcutaneous

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injections of minced tumor preparations. The tumor originated from a hormonally stimulated preneoplastic nodule transplanted to a mouse treated with 7,12-dimethylbenzanthracene (12).

Viruses. MMTV purified from RIII milk was kindly provided by the Biological Carcinogenesis Branch of the National Cancer Institute from stocks supplied by the Meloy Laboratories. The MMTV preparation was purified at the Meloy Laboratories by a combination of rate zonal and isopycnic centrifugation. MMTV (C3H) purified from the culture fluids of the C3H mammary tumor cell line Mm 5 mt/C_1 was obtained from stocks at the Frederick Cancer Research Center.

The viruses obtained from the supernatant fluids of Crandall feline kidney cells infected with MMTV (C3H), MMTV (RIII), and MMTV (GR) have been extensively characterized and have been shown to be characteristic of MMTV and free of murine type C viruses by morphological, antigenic, and biochemical criteria (13).

Migration inhibition studies. Peritoneal exudate cells (PEC) were induced by intraperitoneal injections of 2 ml of 1% oyster glycogen, 48 hr prior to harvest. PEC migration was assayed by an adaptation of the method of Pomales and Ortiz (14) as described by Lopez *et al.* (10). Migration inhibition was calculated from the formula,

$$\% \text{ of migration inhibition} = 1 - \frac{AT}{AC} \times 100,$$

where AT is the area of migration in the presence of test substance and AC is the area in control plates. Thirty percent inhibition of migration was taken as the lower limit of significance.

Lymphocyte transformation assay. Single cell suspensions of spleens were obtained and subjected to water shock to eliminate contaminating red blood cells as previously described (15). The lymphocyte preparations were adjusted to a concentration of 1×10^6 cells/ml in the growth medium described by Click *et al.* (16). MMTV dilutions were added in 25-μl volumes. Cultures were maintained for 3 days in microculture plates (Linbrow, Flow Labs, Rockville, Md.) with 2×10^5 cells per well and were given an 18-hr pulse of tritiated thymidine (0.5 μCi/well). The cells were collected on glass fiber filter paper with distilled water in a Skatron multiple automated sample harvester. The filters were counted for radioactivity and results were expressed as counts per minute in stimulated cultures minus the background counts obtained in cultures of lymphocytes with no MMTV. Unstimulated cultures from lymphocytes from normal mice had 639 ± 118 cpm and those from tumor-bearing mice had 784 ± 139 cpm.

Statistical analysis. Results were analyzed with Student's t test. Probability values are given in the text and tables.

Results. The migration of peritoneal exudate cells from normal and mammary tumor-bearing Balb/cCrgl mice was assessed in the presence of mouse mammary tumor viruses derived from RIII mouse milk and from tissue cultures from spontaneous mammary tumors appearing in C3H mice. As seen in Table I final concentrations of 1, 2, and 4 μg of protein of either kind of MMTV were capable of inhibiting the migration of PEC of normal mice and tumor bearers. However, at every concentration tested, the percentage of migration of PEC in the presence of MMTV derived from C3H tissue cultures was less than that obtained with the same concentration of MMTV purified from RIII milk, indicating higher levels of inhibition of migration with MMTV-C3H antigen(s). These results were statistically significant as determined with Student's t test at probability levels of at least <0.05.

The potential of MMTV-RIII and MMTV-C3H to elicit blastogenic transformation in Balb/cCrgl mice was determined using various concentrations of the viral preparations. In Fig. 1 it can be seen that the incorporation of [^{3}H]thymidine obtained in lymphocyte cultures stimulated with MMTV derived from the C3H strain was consistently higher than that of cultures exposed to MMTV derived from RIII milk. These differences were statistically significant at a $P < 0.05$. In these studies we have included normal and mammary tumor-bearing mice as donors of lymphocytes. In recent studies (17) we have shown

TABLE I. MIGRATION INHIBITION OF PEC FROM NORMAL AND D1-DMBA-3 TUMOR-BEARING Balb/cCrgl MICE IN THE PRESENCE OF MMTV DERIVED FROM RIII MOUSE MILK OR FROM C3H CULTURED MAMMARY TUMOR CELLS

Stimulus	Final concentration (μg)	Normal Balb/cCrgl mice	D1-DMBA-3 tumor-bearing Balb/cCrgl mice
No stimulus	—	100	100
MMTV from RIII milk	4	67.9 ± 1.3*	66.8 ± 1.9*
	2	56.9 ± 2**	57.3 ± 1.5*
	1	64.7 ± 1.3*	65.2 ± 2.4**
MMTV from C3H tissue culture	4	60.3 ± 1.8	61.1 ± 1.7
	2	48.3 ± 2.2	49.7 ± 1.9
	1	59.4 ± 1.6	57.3 ± 1.9

Note. Results are expressed in average percentage of the migration in test cultures relative to that in control without viral antigens. Eight animals were tested per point.

* Significantly different from the migration in the presence of the same concentration of MMTV-C3H ($P < 0.01$).

** Significantly different from the migration in the presence of the same concentration of MMTV-C3H ($P < 0.05$).

that there are no major differences in the responses of these two groups of mice in neither the migration inhibition nor in the lymphocyte transformation assays when they are exposed to MMTV from RIII milk. However, since MMTV derived from C3H tissue culture appears to be more stimulatory in our model systems, it was deemed necessary to compare the effect of the two sources of MMTV in both normal and tumor-bearing mice.

It could be argued that the differences in

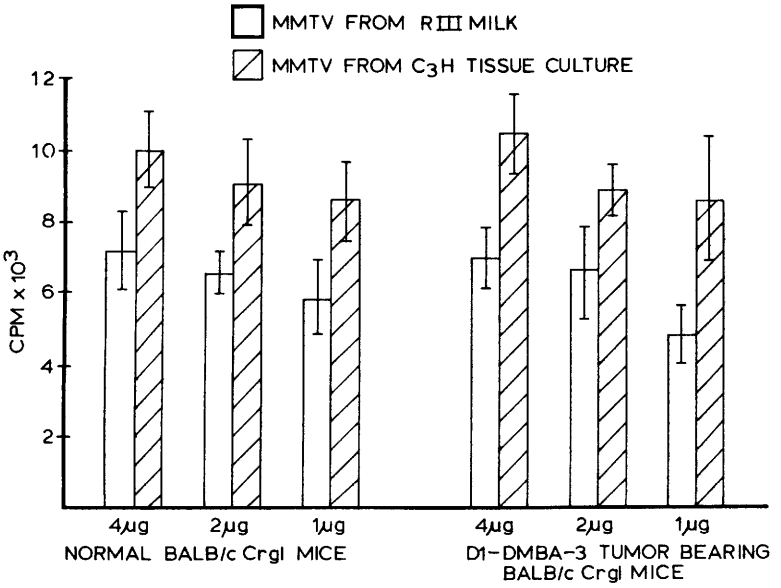

FIG. 1. Blastogenic responses of spleen cells from normal or mammary tumor-bearing Balb/cCrgl mice when stimulated by MMTV antigens derived from RIII mouse milk or from mammary tumor cells from C3H mice in tissue culture. Results are expressed in cpm per well with virus minus the background cpm of nonstimulated spleen cells ± SE. The values obtained with MMTV from RIII milk are significantly different from the ones obtained with MMTV from C3H tissue cultures ($P < 0.05$). These results are a composite of quadruplicate cultures of eight animals per point.

cell-mediated immune responses observed with the two different preparations of MMTV could be due to minor contaminants that were not removed in the viral purification procedure or due to the fact that one preparation was derived from tissue culture fluids and the other from milk. In order to test this hypothesis, MMTV-RIII and MMTV-C3H obtained from the same source, i.e., Crandall feline kidney cells, were tested in the lymphocyte transformation assay. A third preparation of MMTV, originally obtained from GR mice and also grown in the feline tissue culture, was also tested in parallel. Exposure of the spleen cultures to supernatant fluids from uninfected feline cells did not result in any stimulation above the background counts per minute from nonstimulated cultures (639 ± 118 vs 598 ± 122). Other cultures routinely included in our experimental protocol contained, in addition, purified Rauscher leukemia virus at the same protein concentrations. In no case was there any stimulation with this C type particle in our lymphocyte preparations. In Fig. 2, it can be seen that greater incorporations of [^{3}H]thymidine were found in cultures of spleen lymphocytes from Balb/cCrgl mice stimulated with a range of concentrations of MMTV-C3H, than when the same amounts of MMTV-RIII or MMTV-GR were used as stimuli. These results argue against the possibility that the differences in stimulatory potential of MMTV-C3H, MMTV-RIII, and MMTV-GR were due to nonviral components.

Discussion. Evidence has been presented that it is possible to detect type differences among mammary tumor viruses obtained from various mouse strains using cell-mediated immune assays. Previous serological studies have indicated antigenic variations among MMTV isolates using virus neutralization (18), immunization (19), and immunodiffusion techniques (6). However, the significance of these data was difficult to assess since the MMTVs used were not purified and could be contaminated with cellular debris or other host-derived materials.

Using highly purified preparations of

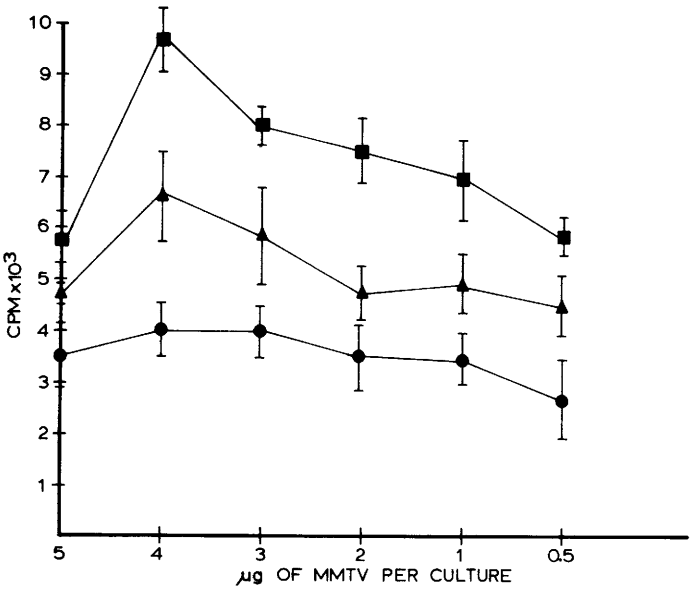

FIG. 2. Incorporation of [^{3}H]thymidine by splenocytes from normal Balb/cCrgl mice stimulated by MMTV derived from the C3H (■) RIII (▲), and GR (●) mouse strains grown in Crandall feline kidney cell cultures. Results are expressed in cpm per well containing virus minus the background cpm of nonstimulated spleen cells ± SE. Counts per minute of the cultures stimulated by MMTV-C3H are significantly different from the cultures containing MMTV-RIII at a probability level of $P < 0.05$ and from the cultures containing MMTV-GR at a P value <0.001. These results are a composite of quadruplicate cultures of 10 animals per point.

MMTVs, Teramoto *et al.* (7) were able to distinguish type- and group-specific antigenic determinants in the major external glycoprotein, gp52, of the mouse mammary tumor viruses of RIII, GR, and C3H mice. These determinants were demonstrated using antisera to the gp52 of MMTV from RIII mice in competitive radioimmunoassays with radioactively labeled virions from the three species mentioned. In subsequent studies, Teramoto and Schlom (8, 20) were able to detect type-specific and group-specific antigenic reactivities for the major internal structural protein of MMTV, p28, and to the virion envelope glycoprotein, gp36.

Recently, Massey *et al.* (21) and Colcher *et al.* (22) have been able to apply the hybridoma technique to obtain monoclonal antibodies which discriminate antigens from the various kinds of mouse mammary tumor virions. Furthermore, they were able to demonstrate the presence of multiple antigenic determinants within the gp52 (21) and the gp36 and p28 (22) of different MMTVs. These epitopes represent type, group, and interspecies determinants.

Differences in the behavior of mammary tumors between the MMTV-expressing strains C3H and RIII mice have been postulated to be due to their respective MMTVs, since they can be perpetuated when the two types of viruses are transferred to mice of identical genotype by foster nursing (23). The differences in bioactivities are expressed as increased numbers of mammary hyperplastic alveolar nodules and higher incidence of mammary tumors in Balb/c female mice foster nursed in C3H mice as compared to those foster nursed in RIII mice (9). An important aspect of those studies was the finding of a higher frequency of lung metastases in the Balb/cfC3H mice. In a recent study (17) we have correlated the presence of a natural *in vitro* immunity to MMTV antigens in Balb/c mice with a certain degree of resistance to implantation with spontaneous mammary tumors expressing MMTV from the C3H strain. The recognition of the antigenic determinants in various MMTV isolates by the lymphoid cells of Balb/cCrgl mice suggests that the differences in bioactivities of MMTV-C3H and MMTV-RIII, could be due in addition to intrinsic viral properties, to host recognition factors that may be involved in the control of tumor development, and in the eventual appearance of metastatic lesions.

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